DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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Compiled by

THE DIVISION OF AIDS

NATIONAL INSTITUTE OF ALLERGY & INFECTIOUS DISEASES

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and

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DRUG RESISTANCE SCREENING ASSAY
NEN Life Science Products RT-Detect™ Method

I. PRINCIPLE

Mutations in the HIV-1 reverse transcriptase (RT) gene have been shown to confer resistance to both classes (nucleoside analogs and non-nucleoside RT inhibitors) of antiviral drugs that affect RT. *In vivo*, nucleoside analogs are taken up by cells and converted to an active form before being incorporated into cDNA and causing premature chain termination. Thus for zidovudine (ZDV), the active form of the drug is ZDV-TP; for didanosine (ddI), it is ddATP. Non-nucleoside drugs must also be taken up by cells, but do not require activation to function and are thought to exert their effects directly upon the viral RT.

Because it has been reported that the RT isolated from ZDV-resistant isolates cannot be distinguished from that isolated from ZDV-sensitive isolates by studying the effect of ZDV-TP on enzymatic activity, it has become customary to determine the drug resistance of viral isolates by examining the effects of a drug on the production of viral proteins in culture. However, it appears that most RT-inhibiting drugs other than ZDV, including both activated nucleoside analogs such as ddATP and non-nucleoside inhibitors such as nevirapine do show differential effects on the activity of RTs from sensitive and resistant isolates. There have also been some recent reports that certain mutations leading to ZDV resistance cause kinetic differences in the enzyme.

The NEN Life Science Products RT-Detect™ assay (NEK-070A) is an end-point method for measuring the amount of RT activity in a sample by quantitating the amount of cDNA produced during the incubation period. When used with the NEN Life Science Products ddI resistance pack (NEK-070I), the RT-Detect™ assay can be employed with an RT-containing viral extract prepared from a single 2 mL co-culture to evaluate the amount of cDNA produced in the absence or the presence of various amounts of drug. This information can be used to calculate a 50% inhibitory concentration (IC_{50}) that numerically represents the degree of resistance of the RT to the drug. Because it includes a heteropolymeric template RNA, the RT-Detect™ kit can be used to evaluate resistance to nucleoside analogs of any base. Like other methods based on RT activity in the presence of drug, this method cannot distinguish between most ZDV-sensitive and -resistant RTs.

II. SPECIMEN REQUIREMENTS

HIV-1 is isolated by conventional co-culture techniques. This method uses 2 mL of supernatant from a 7 day co-culture.

III. REAGENTS

The NEN Life Science Products ddI Resistance Pack (NEK-070I) contains the amount of each component listed below, enough to use with two or more 96-well microplates, depending on the component. If the assay is performed as described here, the ddI Resistance Pack can be used for at least 10-12 specimens. The entire ddI Resistance Pack may be stored at -20^{0}C or below. If desired, Diluent A and 8X Buffer may also be stored at 2 -8^{0}C. Expiration dates are printed on individual component labels and on the kit box.

rHIV-1 reverse transcriptase - One (1) vial containing 50 µL of solution at 10 units/mL.

ddATP, 5 mM - One (1) tube containing 100 µL of 5 mM ddATP in 10 mM Tris-HCl (pH 7.5) with 1 mM EDTA.

Diluent A - One (1) bottle containing 5 mL of 10 mM Tris-HCl (pH 7.5) with 1 mM EDTA.

8X Buffer - One (1) bottle containing 2 mL of 400 mM Tris-HCl (pH 8.0) and 80 mM $MgC1_2$.

The NEN Life Science Products RT-Detect™ 2-plate kit (NEK-070A) contains the amount of each component listed below, enough to perform 192 individual reverse transcription reactions (40 µL scale) and to analyze the products on two 96-well microplates. As described, the kit can be used for 10-12 specimens if 7 levels of drug and a control are run in duplicate. The entire kit should be stored at 2 - 8^{0}C, but it is recommended to store the 20X Plate Wash Concentrate at room temperature to minimize the formation of crystals. Expiration dates are printed on individual component labels and on the kit box.

PEG Solution - One (1) bottle containing 100 mL of solution consisting of 30% (w/v) polyethylene glycol 8000 in 0.4 M NaCl.

Lysis Buffer - Two (2) bottles containing 40 mL of solution consisting of 100 mM Tris-HCl (pH 8.0), 160 mM KCl, 1 mM EDTA, 3 mM dithiothreitol (DTT), 0.3% (v/v) Triton X-100, and 10% (v/v) glycerol.

Template Solution - Four (4) tubes, each containing 1.1 mL of solution containing template RNA, primer, dNTPs, DTT, and an RNase inhibitor.

4X Buffer - Two (2) bottles containing 4 mL of solution consisting of 200 mM Tris-HCl (pH 8.0) and 40 mM $MgCl_2$.

Positive Control DNA - Two (2) tubes containing 150 µL of a synthetic oligonucleotide containing sequences complementary to both the Capture and the Detector Probes. The actual concentration of DNA (between 1000 and 3000 fmol/mL) is printed on the label.

V-Well Microplate - Two (2) 96-well microplates (one molded piece), to be used for RT reactions.

Streptavidin - Coated Microplate - Two (2) 96-well microplates (12 strips of 8 flat-bottom wells), in a re-sealable bag with a desiccant pack, to be used for the ELOSA and Detection reactions.

Hydrolysis Reagent - One (1) bottle containing 4.0 mL of alkaline solution.

Neutralization Reagent - One (1) bottle containing 4.0 mL of phosphate buffer.

Probe Diluent - Two (2) bottles containing 6.0 mL of buffer solution with salts, formamide, protein and detergent.

Probe Solution, 50X - Two (2) tubes containing 110 µL of a 50X concentrate of the HRP-labeled detector probe and the biotin-labeled capture probe.

TMB Substrate - Two (2) bottles containing 10 mL of 3, 3', 5, 5' tetramethylbenzidine (TMB) solution.

TMB Stop Solution - Two (2) bottles containing 10 mL of acidic solution.

Plate Covers - Fourteen (14) adhesive plate covers.

20X Plate Wash Concentrate - One (1) bottle containing 100 mL. Additional 20X Plate Wash Concentrate can be ordered from NEN Life Science Products (Catalog Number FP360).

Distilled or deionized water for diluting 20X Plate Wash Concentrate to 1X.

IV. SUPPLIES AND EQUIPMENT

Lab coat
Gloves
Incubator or oven capable of holding the contents at 37^0C
Automatic or manual microplate washer, vacuum source and trap, hand-held multichannel refilling syringe, or a washing bottle for washing the microplate. Blank strip wells, for use in plate washers with less than a full plate of test wells, are available from NEN Life Science Products on special request
Disposable absorbent bench top paper and paper towels
Vortex mixer
Micropipettor(s) capable of accurately delivering 1 - 1000 µL
Reagent reservoirs for reagent preparation and additions, such as Costar Catalog Number 4870. Due to small reagent volumes, not for use with Reaction Mix or ELOSA Mix
Microplate reader with the capability to read absorbance at 450 nm. Use of a 650 nm reference filter is recommended

Liquid household bleach for inactivation of biohazardous specimens
Polypropylene tubes for preparing dilutions of standards
Centrifuge and tubes for sample processing

V. PROCEDURE

A. Preparation of viral extract

1. Centrifuge 2 mL of supernatant from a 7 day co-culture at 1500 x g for 10 minutes to pellet cells and debris.

2. Add 1 mL of PEG Solution to the supernatant and keep at 4^0C overnight.

3. Centrifuge at 2100 x g for 45 minutes to prepare a viral pellet.

4. Resuspend the pellet in 300 µL of Lysis Buffer. This solution contains the RT from the isolate. If desired, the solution may be stored at -70^0C for at least 1 month before completing the assay.

B. Specific steps for measuring resistance to ddI

Testing for ddI resistance requires running a single RT sample in the presence and absence of ddATP (Ahluwalia, et al., 1987). With the exception of the modifications described below, the assay is identical to that described in the RT-Detect™ kit manual and you must be familiar with that procedure in order to perform a test for ddI resistance. Before running the ddI resistance assay for the first time, review the "Limitations of Procedure" (Section VII).

Differences from the RT-Detect™ kit manual

1. For ddI resistance, the 4X Buffer supplied in the RT-Detect™ kit is not used. Instead, 5 µL of the 8X Buffer from the ddI Resistance pack and 5 µL of a ddATP dilution are added. Thus a reverse transcriptase reaction for ddI resistance contains the following components: 25 µL of Reaction Mix (consisting of 20 µL of Template Solution and 5 µL of 8X Buffer), 5 µL of solution containing a known concentration of ddATP, and 10 µL of a standard RT or a sample containing RT.

2. Because many viral isolates contain too little RT to reliably measure in a 1 hour reaction, the reverse transcription reactions should be incubated for 24 hours.

3. Each RT sample must be run at least in the presence and absence of ddATP. Running a number of ddATP concentrations as described below provides enough data to permit the calculation of an IC_{50} via the median effect equation (Chou and Talalay, 1984; Chou, 1991).

4. Preparation of Solutions for Day 1 - ddI resistance

a. ddATP dilutions

The procedure described below uses 2 strips of wells per sample to generate a 7-point inhibition curve in duplicate. This amount of information permits the calculation of an IC_{50}. To run fewer points, or compare to a cutoff value, adjust the number and the preparation of ddATP dilutions accordingly to prepare the necessary volume.

To prepare enough of each ddATP dilution for one full plate of wells, make a series of ddATP dilutions in Diluent A and label them as shown in the following table.

Label	Drug Added	Diluent A	µM in Tube	µM in Assay
H	40 µL of ddATP, 5M	210 µL	800	100
G	50 µL of H	150 µL	200	25
F	50 µL of G	150 µL	50	6.25
E	100 µL of F	100 µL	25	3.12
D	100 µL of E	100 µL	12.5	1.56
C	50 µL of D	150 µL	3.12	0.39
B	50 µL of C	150 µL	0.78	0.09
A	None	100 µL	0	0

b. Reaction Mixes - ddI

Set up 8 microcentrifuge tubes labeled A', B', C', D', E', F', G' and H' to correspond to the tubes A through H that hold the ddATP dilutions. For each RT sample to be run, add:

48 µL of Template Solution
12 µL of 8X Buffer
12 µL of the correspondingly-labeled ddATP solution to a labeled microcentrifuge tube. Thus, for an experiment with 3 samples and the rHIV RT control, Reaction Mix A, for example, will contain 192 µL of Template Solution, 48 µL of 8X Buffer and 48 µL of ddATP Solution A.

C. Specific steps for measuring resistance to Nevirapine

Although the ddATP and Diluent A are not used, it is still convenient to use the 8X Buffer and rHIV-1 RT from the ddI Resistance Pack as a supplement to the RT-Detect™ kit to measure resistance to nevirapine. At the time of this writing, nevirapine has just become commercially available and it is not included in the kit. With the exception of the modifications described below,

the assay is identical to that described for ddI resistance and you must be familiar with that procedure in order to perform a test for nevirapine resistance. Before running the nevirapine resistance assay for the first time, review the "Limitations of Procedure" (Section VII).

Differences from measuring ddI resistance

1. Due to solubility limitations, Nevirapine must be dissolved and diluted in DMSO, not in Diluent A.

2. In order to minimize the amount of DMSO introduced into the RT reactions (DMSO can be inhibitory in large enough amounts) only 2 µL of Nevirapine dilution is added to each Reaction Mix. This alters the amounts of solutions added to prepare the Reaction Mixes.

3. Each RT sample must be run at least in the presence and absence of nevirapine. Running a number of Nevirapine concentrations as described below provides enough data to permit the calculation of an IC_{50} via the median effect equation (Chou and Talalay, 1984; Chou, 1991).

4. Preparation of Solutions for Day 1 - Nevirapine resistance

   a. Nevirapine dilutions

   The procedure described below uses 2 strips of wells per sample to generate a 6-point inhibition curve in duplicate wells. This amount of information permits the calculation of an IC_{50}. To run fewer points, or compare to a cutoff value, adjust the number and the preparation of Nevirapine dilutions accordingly to prepare the necessary volume.

To prepare enough of each Nevirapine dilution for one full plate of wells, prepare a 100 mM stock solution of Nevirapine in DMSO and prepare further dilutions in DMSO as shown in the following table.

Label	Drug Added	DMSO	µM in Tube	µM in Assay
G	18 µL of 100 mM Nevirapine	82 µL	18000	375
F	10 µL of G	90 µL	1800	37.5
E	10 µL of F	90 µL	180	3.75
D	10 µL of E	90 µL	18	0.375
C	10 µL of D	90 µL	1.8	0.038
B	10 µL of C	90 µL	0.18	0.004
A	None	90 µL	0	0

   b. Reaction Mixes - Nevirapine

Set up 8 microcentrifuge tubes labeled A', B', C', D', E', F', G' and H' to correspond to the tubes A through H that hold the nevirapine dilutions. For each RT sample to be run, add:

48 µL of Template Solution
12 µL of 8X Buffer
10 µL of water
2 µL of the correspondingly-labeled Nevirapine solution to a labeled microcentrifuge tube. Thus, for an experiment with 3 samples and the rHIV RT control, Reaction Mix A, for example, will contain 192 µL of Template Solution, 48 µL of 8X Buffer, 40 µL of water and 8 µL of Nevirapine Solution A.

D. Assay Day 1

1. Preparation of Solutions

a. rHIV-1 RT

Prepare a standard rHIV-1 RT dilution as follows: Add 5 µL of starting RT (supplied at 10 units/mL) to 95 µL of Lysis Buffer. Mix, and then add 10 µL of the dilution to 240 µL of Lysis Buffer to give a solution containing 0.02 units/mL of RT. Use this final solution (0.02 units/mL) as the standard drug-sensitive RT dilution for the test.

b. Samples

Culture supernatant samples (2 mL) are processed with PEG to give a viral pellet and resuspended in Lysis Buffer (300 µL) as described above. The Lysis Buffer samples are used directly without dilution for resistance assays.

c. Drug Solutions and Reaction Mixes. Described above.

2. Procedure

a. For each sample or RT standard to be run, add 30 µL of the appropriate Reaction Mix A'- H' to 2 wells in the correspondingly-labeled row of the "v"-well plate. That is, Reaction Mix A' goes into plate row "A", etc. Be careful not to confuse Reaction Mix A' with drug dilution A, etc. Note that there are only 7 Reaction Mixes described for Nevirapine, so one row will either be empty, or can be used to run additional standards or samples.

b. Add 10 µL of each sample or RT standard to 16 wells in two columns of the plate. Each RT sample should be in 2 columns of wells and each plate

row should have a different concentration of drug. Cover the plate, sealing carefully to minimize evaporation. Incubate for 24 hours at 37^0C.

E. Assay Day 2

1. Preparation of Solutions

a. ELOSA Mix

For each sample to be run, add 18 µL of Probe Solution and 882 µL Probe Diluent to a conical centrifuge tube to prepare ELOSA Mix.

b. Plate Wash

Prepare Plate Wash by warming 20X Plate Wash Concentrate at 37^0C if necessary to dissolve any crystals and then diluting it to 1X with distilled or deionized water. About 500 mL of 1X Plate Wash is needed to prime an automated washer and run one microplate. 1X Plate Wash should be made fresh daily.

2. Procedure

a. Denaturation (and Hydrolysis of Template RNA)

1) Remove the plate cover and add 10 µL of Hydrolysis Reagent to each well. Cover the plate and incubate 15 minutes at 37^0C.

2) Add 10 µL of Neutralization Reagent to each well.

b. Sandwich Hybridization

1) Transfer all the liquid from each v-well to a corresponding well in the flat-bottom streptavidin-coated plate. If the plates were tightly sealed, there should be about 40-50 µL of liquid in each well. At this step, volume variations do not affect the assay results.

2) Add 50 µL of ELOSA Mix to each well. Cover, and incubate for 2 hours at 37^0C.

c. Detection

1) Wash the plate 6 times with 1X Plate Wash.

2) Add 100 µL of TMB Substrate to each well. Cover, and incubate 1 hour at room temperature.

3) Add 100 µL of Stop Solution to each well. Within 1 hour, read the absorbance twice, once at 450 nm and once at 490 nm, using a 650 nm reference filter in each case if possible.

VI.. CALCULATION OF IC_{50}

A. Signal Requirements

1. If the absorbance of a sample in row A (no drug) is less than 0.500, there is insufficient RT in the sample to give a reliable IC_{50}. Before running the resistance assay on a large set of samples, it may be wise to check the total RT activity of each sample in a preliminary experiment to be sure it is sufficient to perform the test. In a test with 25 clinical isolates, 3 (12%) had insufficient activity to perform the test.

2. If the absorbance of a sample in row A is off-scale at 450 nm (for example, greater than 4.000 on a Molecular Devices plate reader), the absorbance reading at 490 nm (if it is on-scale) may be used to calculate the results. If the absorbance reading at 490 nm is off-scale, the remaining RT sample should be diluted 20-fold with Lysis Buffer and used to repeat the test.

3. If the absorbance of a sample in row A is between 0.500 and 4.000 (or other limit of the plate reader used) at 450 nm, use the readings at 450 nm to calculate the results.

B. Calculations

1. Average the absorbance values for replicate tests to get the mean absorbance for each.

2. Divide the mean absorbance values for each RT sample by the mean absorbance of the same reaction in row A (no drug) to normalize the results for the amount of RT activity in the sample.

3. Plot the normalized mean absorbance for each RT reaction on the y-axis versus the log of the concentration of drug (µM) in that reaction on the x-axis. Examining the plot can help to identify and delete outlying points. Sometimes one of the two duplicate wells is an outlier, but the other well can be used.

4. If a sufficient number of drug concentrations were run to obtain a reasonable curve, commercially available software such as Systat (Systat, Inc.) or JMP (SAS Institute, Inc.) may be used to calculate an IC_{50} (the concentration of drug that inhibits the activity of the RT sample by 50% relative to the activity in the absence of drug) by the median effect equation (Chou and Talalay, 1984; Chou, 1991):

$$FA = 1 - \frac{1}{1+(Y/Dose)^m}$$

where: FA = (0.01)(% reduction from untreated control)
DOSE = drug (µM) in the RT reaction
$Y = IC_{50}$
M = slope

VII. QUALITY CONTROL

The user must establish any cut-off value to be used in distinguishing drug-sensitive from drug-resistant isolates.

VII. Limitations of Procedure

A. Safety Considerations

In the US only, the components of 8X Buffer are considered to be irritating to eyes and skin.

HYDROCHLORIC ACID; 2-CHLOROACETAMIDE;
FORMAMIDE; POTASSIUM HYDROXIDE

DANGER

CONTAINS MATERIALS THAT CAN BE FATAL IF INHALED, SWALLOWED, OR ABSORBED THROUGH SKIN. CONTAINS CORROSIVE MATERIALS THAT CAN CAUSE SEVERE BURNS TO EYES, SKIN AND ALL OTHER BODY TISSUES. CONTAINS MATERIALS THAT ARE POSSIBLE MUTAGENS AND TERATOGENS. TARGET ORGANS: Skin, Eyes, Male Reproductive Systems, Thyroid, Ovaries, Fallopian Tubes, Lungs, Gastrointestinal Tract, Blood, and Mucous Membranes. Do not get in eyes, skin, or clothing. Avoid breathing vapors. Use adequate ventilation. Wash thoroughly after handling. FIRST AID: In case of contact, immediately flush eyes and skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes.

1. Handle assay specimens, especially those of human origin, as if capable of transmitting an infectious agent (NIH Guidelines, BSL 2 or higher, as necessary, [U.S. Department of health and Human Services, 1988]).

2. Wipe spills promptly with 1% sodium hypochlorite solution (1:5 dilution of liquid household bleach). Contaminated materials should be disposed of as biohazardous waste.

3. Do not pipette by mouth. Avoid splashing and generation of aerosols.

4. Do not eat, smoke, or drink in areas in which specimens or kit reagents are handled.

5. Wear disposable gloves throughout the test procedure. Dispose of the gloves as biohazardous waste. Thoroughly wash hands afterward.

B. Performance Considerations

1. This method is not useful for determining resistance to ZDV.

2. **Prevent RNase Contamination** – The template RNA provided in the RT–Detect™ kit can be degraded by RNase. The Template Solution contains an RNase inhibitor, so RNase contamination will normally not be a problem in the assay. Two simple precautions can minimize RNase contamination if employed during the steps prior to the addition of the Hydrolysis Reagent. (Since the Hydrolysis Reagent destroys RNA, it is not necessary to take precautions to avoid RNase contamination in subsequent steps.) Wearing gloves at all times when working with RNA will prevent RNase contamination originating from the skin. Use of sterile disposable polypropylene plasticware, especially pipette tips, is recommended to prevent RNase contamination originating from labware.

3. There is not a sufficient amount of the components of the Reaction Mix and ELOSA Mix in the kit to allow preparing enough excess to permit the use of a trough and a multichannel pipette for addition of these reagents. The use of a repeater pipettor is recommended for these steps.

4. Before or after adding it to the microplate, avoid placing the TMB Substrate in proximity to a container of diluted bleach, such as is commonly used for decontamination in biosafety hoods. Vapors from the bleach solution can cause color development by the TMB, even if the plate is covered and kept in a hood.

5. Do not allow the microplate wells to dry out once the assay has begun.

6. Do not use kit components beyond the expiration date. This date is printed on individual component labels and on the kit box.

7. Do not substitute reagents from other kits. Reagents have been optimized for performance with each kit lot. Dilution or alteration of reagents may result in loss of sensitivity or other undesirable changes in kit performance.

8. Do not interchange vial or bottle caps and stoppers: this will lead to cross-contamination of reagents. Use specific reservoirs for specific reagents, and use

clean pipettes or pipette tips for each reagent. Cross-contamination of samples could cause false results.

9. Incubation times or temperatures other than those specified may give erroneous results.

C. General Precautions

1. Microplate washing may be manual, automated or semi-automated, but must be carried out with care to ensure optimal performance of the assay. Plate washing equipment must be properly adjusted, cleaned and maintained.

   a. Automatic Microplate Washer – Set the fill volume to 300 µL/well. Prime the instrument with Plate Wash. Use two 3-cycle washes. After the initial 3-cycle wash, invert the plate and firmly strike it against an absorbent surface. Rotate the plate 180 degrees and repeat.

   b. Manual Microplate Washer – Wash six (6) times, using 300 µL/well per wash. Fill the entire plate, then aspirate in the same order.

   c. Hand-held Syringe or Wash Bottle – Wash six (6) times, using 300 µL/well per wash (filling the wells). Blot the plate upside-down between washes.

   After the final wash, invert the microplate and firmly strike it on an absorbent surface. Visually check that all wells are empty.

2. When inverting the microplate to decant or blot, press the side tabs of the frame inward to prevent the strips from falling out. As an additional precaution, it is advisable to apply tape to the edges of the strips.

3. The Template Solution and reagents containing enzymes (the samples to be tested, any RT standards employed, ELOSA Mix and Probe Solution, 50X) should be kept cold until used.

E. Other Issues

1. DNA-dependent DNA polymerases

Cell extracts may contain DNA-dependent DNA polymerase activities that are distinct from RT, but can generate cDNA from certain RNA homopolymers. The use of a heteropolymeric RNA template in the RT-Detect™ assay prevents these activities from being expressed. Thus, unlike assays which use homopolymer templates, it is *not* necessary with RT-Detect™ to run a parallel reaction using a DNA template to prove that the enzyme activity observed is due to the presence of reverse transcriptase.

2. Proteases

If protease activity in the sample is a concern, 2 mM phenylmethyl sulfonyl fluoride (PMSF) may be added during sample processing with no effect on the RT-Detect™ assay.

F. Troubleshooting

1. The Positive Control DNA from the RT-Detect™ kit can be used as a control for the detection reactions. (Because it is added directly and not generated by reverse transcriptase during the assay, it cannot act as a control for the reverse transcription reactions.) Running a standard curve of the Positive Control DNA as described in the RT-Detect™ kit manual can be useful in trouble-shooting assay problems. Such a standard curves obtained should be nearly linear if the protocol is strictly followed. Variations from the protocol may increase the non-linearity of the standard curve. For a non-linear result, point-to-point or non-linear curve fitting methods should be used.

2. Poor duplicates may indicate inaccurate pipetting or insufficient washing. If all instructions for the preparation and use of Plate Wash were carefully followed, such results may indicate a need for plate washer maintenance.

   Alternatively, poor duplicates may be caused by insufficient mixing after addition of the TMB Stop Solution. This type of problem can be identified by re-reading the absorbance 15 - 60 minutes after addition of the TMB Stop Solution. Insufficiently mixed solutions will equilibrate over this time period and give increased signal and better duplicates.

3. A Substrate Blank Well (a microplate well which is left empty until the TMB Substrate and Stop Solution are added), read against air, should read less than or equal to 0.1 absorbance unit when read at 450 nm with a 650 nm reference filter. A significantly higher value may indicate deterioration of the TMB Substrate solution, but when readings are corrected by subtracting the absorbance of the 0 fmol/mL Positive Control DNA standard or the 0 units/mL RT standard, valid assay results will usually be obtained.

VIII. REFERENCES

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